Long-Term Exposure to Tamoxifen Induces Hypersensitivity to Estradiol

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ABSTRACT

In women with hormone-dependent breast cancer, tamoxifen (TAM) frequently induces tumor regression, but regrowth occurs with continuation of antiestrogen therapy. Studies of breast xenografts in nude mice suggest that this secondary resistance to TAM may reflect the development of enhanced sensitivity to the estrogenic properties of TAM. In the current study, we examined the hypothesis that TAM could also induce a state of hypersensitivity to estradiol (E2) itself. Oophorectomized nude mice with MCF-7 cell xenografts received 25-mg implants of TAM [long-term TAM treated (LTTT) mice] or cholesterol (C-MCF-7) over a 5-month period (phase 1). The LTTT group regressed to a lesser extent than did C-MCF-7 tumors. After 4 months of TAM exposure, the LTTT tumors begin to regrow, as did the C-MCF-7, as assessed by slope analysis. At 5 months, TAM or vehicle implants were removed, and the LTTT and C-MCF-7 subgroups were given vehicle or two doses of E2 to test estrogen sensitivity (phase 2). We used our “E2 clamp” technique to maintain levels of plasma E2 at either 1.25 or 20 pg/ml. Neither group responded to the very low concentrations of E2 (1.25 pg/ml) or vehicle. The LTTT tumors but not C-MCF-7 tumors exhibited a growth response on exposure to 20 pg/ml E2 during 7 weeks, as demonstrated with mixed models analysis. These studies provide evidence that long-term TAM exposure enhances sensitivity to the estrogenic effects of TAM and also to E2 itself.

INTRODUCTION

Women with hormone-dependent breast tumors respond to oophorectomy with tumor regression lasting 12–18 months on average but then invariably experience tumor regrowth. This surgical maneuver causes plasma estradiol (E2) to fall from average levels of 150 pg/ml to approximately 10 pg/ml. Administration of an aromatase (estrogen synthase) inhibitor during the regrowth phase causes a further fall in E2 to practically undetectable levels (1–2 pg/ml) and secondary tumor regression. These data suggested that long-term exposure to low E2 levels causes tumors to adapt and become hypersensitive to the stimulatory effects of E2. Based on these clinical observations, we have conducted a series of studies using a long-term estrogen-deprived MCF-7 breast cancer model system to mimic the effects of oophorectomy on serum E2 levels. With both in vitro and in vivo observations, we have directly confirmed the development of “adaptive hypersensitivity” in this model system (1–3).

Postmenopausal women receiving tamoxifen (TAM) long term for breast cancer also experience initial tumor regression followed 12–18 months later by regrowth (4, 5). Studies in nude mouse xenograft models demonstrated that regrowth was associated with development of enhanced sensitivity to the estrogenic properties of TAM (6, 7). It has been clearly shown that aromatase inhibitors cause secondary tumor regressions in women relapsing on TAM. We postulated that TAM may have induced a state of hypersensitivity to E2 in these patients and that the aromatase inhibitors were able to lower E2 levels below those required for tumor stimulation. This hypothesis would provide a rationale why aromatase inhibitors remain effective after patients become secondarily resistant to TAM.

To test our hypothesis about TAM induction of hypersensitivity to E2, we exposed MCF-7 xenografts to TAM for a 5-month period and then tested their level of sensitivity to E2. Long-term TAM-treated (LTTT) tumors responded to low doses of E2 with a stimulatory response, whereas vehicle-treated tumors did not. In contrast, the uteri of LTTT animals exhibited a reduced sensitivity to E2. Our data suggest that long-term exposure to TAM induces a state of adaptive hypersensitivity to estrogen in breast tumors. These results could explain mechanistically why women respond to aromatase inhibitors after developing apparent resistance to TAM.

MATERIALS AND METHODS

We transplanted wild-type MCF-7 breast cancer cells into oophorectomized nude mice and used these animals as our model system. Female, 4–5-week-old, athymic nude mice (n = 39), obtained from Charles River Laboratories, Inc. (Willington, MA), were housed in a pathogen-free environment and treated according to NIH guidelines for the care and use of animals. Oophorectomy was performed under anesthesia a week before cell inoculation. Cultured cells were removed from plates by scraping followed by resuspension in phenol red-free Matrigel (10 mg/ml; Becton Dickinson, Bedford, MA). A total of 5 million MCF-7 cells were injected into each of two identical
contralateral sites on the flanks of each mouse. The xenografts were allowed to become established for 4 weeks while mice received 2 mg of E2 delivered by E2 pellet from Innovative Research of America (Sarasota, FL). After 4 weeks, E2 capsules were removed, and animals were divided into two groups. One group (21 mice) received 25-mg implants of TAM (TAM citrate; Sigma), and resulting tumors were called LTTT. The other group (18 mice) was given implants containing cholesterol as vehicle (Steraloids, Wilton, NH) and called control MCF-7 tumors (C-MCF-7). After 5 months (that is, in the end of phase 1 of experiment), TAM or vehicle implants were removed from all animals. The LTTT and C-MCF-7 groups were then further subdivided into three groups [with seven (TAM) or six (cholesterol) treated animals in each group]. These subgroups received implants with either vehicle alone or E2 “clamped” at a plasma concentration of 1.25 pg/ml or E2 at 20 pg/ml. We have previously described and validated the E2 clamp method, which delivers the desired concentration of E2 at steady-state levels during the time of administration (2). Tumor growth curves were plotted in these animals during the next 7 weeks (phase 2). Tumor volumes were estimated according to the formula \( \frac{4}{3} \pi r^2 \) \( r_{1/2} \) (short axis; \( r_{2/2} \) long axis) and converted into natural logarithms. At the end of the 7-week period, animals were killed, and tumor and uterine weights were measured.

Repeated measures models were used for the analysis of tumor volumes over time (8). Tests for statistical significance were carried out using F-tests based on the Kenward-Roger (1997) approximation (9). Nonparametric tests were used to compare tumor weight and uterine weight at the time of sacrifice.

**RESULTS**

E2 administration over a 1-month period was required for establishment of viable xenografts. Measurements of tumor volume initially began on removal of E2 and start of treatment with either TAM or vehicle. During long-term exposure to TAM or the cholesterol vehicle (phase 1), the LTTT group of tumors regressed to a lesser extent during the first 8 weeks than did C-MCF-7 tumors (Fig. 1). After 14 weeks of TAM exposure, the LTTT tumors began to regrow (Fig. 1) in a manner described previously by Gottardis and Jordan (6) and Osborne et al. (7). The slope for weeks 0–14 was \(-0.032\), and the slope for weeks 15–21 was \(+0.019\) \((P = 0.011\) for the difference in slopes). This indicated a statistically significant regrowth during the later period. The vehicle-treated tumors also regrew from weeks 15 to 21. For the latter (vehicle) group, the slope was \(-0.039\) for weeks 0–14 and \(+0.016\) for weeks 15–21 \((P < 0.001\) for the difference between slopes). When comparing the regression or regrowth phases in the LTTT and C-MCF-7 groups, one can see that the mean tumor profiles are significantly different in the two groups \((P = 0.02)\), and this difference is due mainly to an initial drop in tumor volumes at week 1 in the vehicle-treated group.

An increase in sensitivity of breast cell growth to E2 in the LTTT animals was selective for breast tissue. This conclusion was based on the observation that uterine weight in LTTT animals responded to E2 to a lesser extent than that in C-MCF-7 animals. For example, the highest E2 dose stimulated the uterus in LTTT animals from 12.6 to 69.7 mg, whereas the same dose
increased uterine weight in C-MCF-7 animals from 18.7 to 151.8 mg (Table 1; Fig. 4).

**DISCUSSION**

Our prior studies and those of others suggested that human breast tumors exhibit the property of plasticity and undergo adaptation in response to a low-estrogen milieu. This results in the development of hypersensitivity to E2, a phenomenon that we have termed "adaptive hypersensitivity." This concept provides a mechanistic explanation for the regrowth of breast tumors after surgical oophorectomy in women and the secondary responses to subsequent use of aromatase inhibitors. The present experimental study extends these data by demonstrating that long-term exposure to TAM also causes enhanced responsiveness to low concentrations of E2. The levels of E2 used in this study were equivalent to those found in menopausal women, and the observations can then be considered to reflect physiological conditions.

**Table 1**  Tumor and uterine weight (mean ± SE) in mice at the end of the experiment

<table>
<thead>
<tr>
<th>Weight (mg)</th>
<th>Vehicle</th>
<th>E2 (1.25 pg/ml)</th>
<th>E2 (20 pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor</td>
<td>C-MCF-7</td>
<td>LTTT</td>
<td>C-MCF-7</td>
</tr>
<tr>
<td>Uterus</td>
<td>24.6 ± 5.0</td>
<td>39.0 ± 11.6</td>
<td>23.4 ± 3.4</td>
</tr>
</tbody>
</table>

*E2, estradiol "clamped" on two different blood concentrations (1.25 pg/ml and 20 pg/ml); LTTT, long-term tamoxifen treatment; C-MCF-7, placebo group.

*Difference between placebo and tamoxifen-treated groups is significant (P < 0.05).
Several investigators demonstrated previously that long-term exposure to antiestrogens causes human breast cancer cells to adapt and change their properties. For example, Horwitz et al. (10) showed that in cells treated with nafoxidine for 12 days, the rate of induction of progesterone receptors by E\textsubscript{2} exceeds the level of this parameter in control cells. Gottardis and Jordan (6) as well as Osborne et al. (7) demonstrated that exposure to TAM for 6–12 months causes this antiestrogen to exert agonistic effects on breast cancer xenografts. During more prolonged exposure to TAM for up to 7 years, serially transplanted MCF-7 xenografts further alter their responses to E\textsubscript{2}. Tumors previously stimulated with estrogen respond after several years of TAM exposure by undergoing apoptosis in response to this estrogen (11). We have postulated that this later mechanism might explain why women who are 10–20 years postmenopausal respond to the synthetic estrogen diethylstilbestrol with breast tumor regression (3).

An important finding in the present study is that long-term TAM exposure induced hypersensitivity to E\textsubscript{2} in breast but reduced sensitivity to it in the uterus. The mechanism for this differential adaptive process is not currently known but could relate to up-regulation of growth factor pathways in breast (12, 13). Another explanation could involve an increase in levels of a mutant form of estrogen receptor hypersensitive to the estrogen action (14). Presumably, a more important and frequent cause of increased sensitivity to E\textsubscript{2} is associated with the adaptive changes in tissue responses during long-term TAM treatment. This adaptive reaction could be based on combined alterations in nongenomic or genomic effects of estrogen. The latter might involve increased signaling from peptide growth factors and act via activation of Ras-mitogen-activated protein kinase cascade (3, 12, 13). Additional studies are required to precisely define the mechanisms involved.

An interesting observation in these studies is that TAM appeared to cause a lesser degree of tumor regression initially than did deprivation of E\textsubscript{2}. This observation is apparent on inspection of prior data published by Osborne et al. (7) and Gottardis and Jordan (6). This finding likely reflects some degree of estrogen agonistic activity and could mimic the situation in primary resistance to TAM in patients. Later, the tumors are actually stimulated to grow by TAM, which could mimic secondary TAM resistance. This observation might be considered to represent a form of hypersensitivity to the estrogenic effects of TAM. From a practical point of view, these observations lead to the conclusion that approaches specifically limiting hyper-responsiveness to estrogen warrant additional studies. As underlined by Osborne et al. (15), growth factor inhibitors such as Iressa might prolong the duration of responsiveness to TAM. These may act by abrogating the estrogenic effect of TAM induced by growth factor overexpression.

From clinical observations, at least a third of breast cancer patients with estrogen receptor-positive tumors do not respond initially to TAM treatment. This can be defined as de novo or primary resistance. Other patients develop resistance to TAM during the course of long-term treatment (secondary or acquired resistance; Ref. 4). Several mechanisms explaining this phenomenon have been suggested (16, 17), and the search for other more satisfying concepts continues at an increasing pace. In particular, the assumption was put forward that processes resulting in tumor regrowth in response to TAM as an estrogenic agonist and consequences of the post-therapeutic loss of estrogens in breast cancer tissue could potentially be mechanistically linked (5). This hypothesis received partial support when it was convincingly demonstrated recently that in breast cancer cells subjected to long-term estrogen deprivation, hypersensitivity to E\textsubscript{2} developed (1). Increased sensitivity to estrogen is also characteristic for long-term estrogen-deprived cells transplanted into nude mice (2, 11).

We recognize that estrogen deprivation in vitro and exposure to antiestrogens such as TAM may not provide an equivalent stimulus to the process of adaptation. Additional studies are required to compare what adaptive changes occur in common and disparately in long-term E\textsubscript{2}-deprived cells versus long-term TAM-treated cells. This problem deserves special study with cells and other models subjected to the effect of TAM. The results of such studies performed with wild-type MCF-7 cell xenografts treated long-term with TAM in vivo are presented here. As demonstrated, such xenografts exhibit an enhancement of tumor growth on exposure to E\textsubscript{2}, and this stage of adaptive
hypersensitivity to E2 follows the stage of increased sensitivity to the agonistic effect of TAM.

A potential pitfall in this study is the effects of residual TAM remaining in tumor cells for the 7 weeks after removal of TAM implants. Agonistic effects of this agent on breast could act in an additive fashion with exogenous E2. In addition, antagonistic effects of residual TAM on the uterus could partially inhibit the effects of E2. If correct, such effects would confound the interpretation of our data. We consider these possibilities unlikely because the TAM-pretreated tumors regressed after removal of TAM, indicating a lack of residual agonistic effect on tumor growth (Fig. 3). In addition, the half-life of TAM in blood is approximately 11 days, and levels should have declined by >94% by 7 weeks. At this time point, tumors were still growing in response to 20 pg/ml E2 in the TAM-pretreated group.

Our observation of development of hypersensitivity in response to TAM but not E2 is of interest. Using an in vitro model, the time required to develop hypersensitivity in response to E2 deprivation is 6–24 months, and reversion to wild type on re-exposure to E2 occurs in the same time frame (18). Based on this, we postulate that development of hypersensitivity in response to TAM pre-exposure occurs more rapidly than that induced by E2 deprivation. Additional studies are required to demonstrate this directly.

REFERENCES

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